Bile acid sequestration by cholestyramine mitigates FGFR4 inhibition-induced ALT elevation

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Abstract: The FGF19-FGFR4- Klotho (KLB) pathway plays an important role in the regulation of bile acid (BA) homeostasis. Aberrant activation of this pathway has been described in the development and progression of a subset of liver cancers including hepatocellular carcinoma (HCC), establishing FGFR4 as an attractive therapeutic target for such solid tumors. FGF401 is a highly selective FGFR4 kinase inhibitor being developed for HCC, currently in Phase I/II clinical studies. In preclinical studies in mice and dogs, oral administration of FGF401 led to induction of Cyp7a1, elevation of its peripheral marker 7alpha-hydroxy-4-cholesten-3-one (C4), increased BA pool size, decreased serum cholesterol and diarrhea in dogs. FGF401 was also associated with increases of serum aminotransferases, primarily alanine aminotransferase (ALT), in the absence of any observable adverse histopathological findings in the liver, or in any other organs. We hypothesized that the increase in ALT could be secondary to increased BAs and conducted an investigative study in dogs with FGF401 and co-administration of the BA sequestrant cholestyramine (CHO). CHO prevented and reversed FGF401-related increases in ALT in dogs in parallel to its ability to reduce BAs in the circulation. Correlation analysis showed that FGF401-mediated increases in ALT strongly correlated with increases in taurolithocholic acid (TLCA) and taurodeoxycholic acid (TDCA), the major secondary BAs in dog plasma, indicating a mechanistic link between ALT elevation and changes in BA pool hydrophobicity. Thus, CHO may offer the potential to mitigate elevations in serum aminotransferases in human subjects that are caused by targeted FGFR4 inhibition and elevated intracellular BA levels.

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Title page

Bile acid sequestration by cholestyramine mitigates FGFR4 inhibition-induced ALT elevation

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Abstract

The FGF19-FGFR4-βKlotho (KLB) pathway plays an important role in the regulation of bile acid (BA) homeostasis. Aberrant activation of this pathway has been described in the development and progression of a subset of liver cancers including hepatocellular carcinoma (HCC), establishing FGFR4 as an attractive therapeutic target for such solid tumors. FGF401 is a highly selective FGFR4 kinase inhibitor being developed for HCC, currently in Phase I/II clinical studies. In preclinical studies in mice and dogs, single or repeated doses of FGF401 led to induction of Cyp7a1 in the liver, the rate-limiting enzyme in BA biosynthesis, and elevation of its peripheral marker 7alpha-hydroxy-4-cholesten-3-one (C4), resulting in increased BA pool size, decreased serum cholesterol and diarrhea in dogs. FGF401 was also associated with increases of serum aminotransferases, primarily alanine aminotransferase (ALT), in mice and dogs in the absence of any observable adverse histopathological findings in the liver, or in any other organs. We hypothesized that the increase in ALT could be secondary to increased BAs and conducted an investigative study in dogs with FGF401 and co-administration of the BA sequestrant cholestyramine (CHO) to test this hypothesis. Here we show that co-administration of CHO with FGF401 prevented and reversed FGF401-related increases in ALT in dogs in parallel to its ability to reduce BAs in the circulation. BA profile analysis revealed that effects of BA sequestration were most pronounced for secondary BAs of high hydrophobicity. Indeed, correlation analysis showed that FGF401-mediated increases in ALT strongly correlated with increases in TLCA and TDCA, the major secondary BAs in dog plasma, making a mechanistic link between ALT elevation and changes in BA pool hydrophobicity plausible. These data therefore confirm our hypothesis that the increase in ALT with FGF401 is likely secondary to BAs increases and can be prevented by CHO. In human subjects, CHO may offer the potential to mitigate elevations in serum aminotransferases that are caused by targeted FGFR4 inhibition and elevated intracellular BA levels, in addition to its established role in diarrhea management.

Introduction

The role of FGF19/FGFR4/KLB in bile acid homeostasis. FGF401 is a highly selective and potent FGFR4 inhibitor through binding of the kinase domain adenosine triphosphate (ATP) binding-site of FGFR4, a member of the fibroblast growth factor receptor (FGFR) family. FGF19, which signals through FGFR4 and its co-receptor βKlotho (KLB), both commonly expressed in the liver (1), plays a key role in the regulation of bile acid (BA) homoeostasis (2) and has been associated with liver tumorigenesis (3), leading to a rising interest in inhibiting the FGF19-FGFR4 signaling axis in hepatocellular carcinoma (HCC) (4, 5).

The expression of FGF19 is induced in the ileum in response to BAs released into the intestinal lumen after feeding. Intestinally reabsorbed BAs activate the nuclear transcription factor farnesoid X receptor (FXR) within enterocytes, which controls the transcriptional regulation of FGF19. FGF19 is released into the portal vein and circulates to the liver where FGFR4 and KLB are highly co-expressed. Binding of FGF19 to the FGFR4-KLB complex leads to activation of a signal transduction cascade within the liver that results in the repression of Cyp7a1 transcription, the rate-limiting enzyme for BA synthesis (2). Cyp7a1 converts cholesterol to 7-alpha-hydroxycholesterol, which is further oxidized to 7alpha-hydroxy-4-cholesten-3-one (C4), a stable BA intermediate and peripheral blood marker for hepatic Cyp7a1 activity (6). The pathway subsequently drives the formation of the two primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA) as illustrated in Figure 1 (7). Thus, BA synthesis represents the major catabolic pathway of cholesterol degradation. Within the hepatocyte, BAs are conjugated with glycine (G) or taurine (T) and transported into the bile canaliculi by BA transporters such as BSEP. In dogs, T-conjugation predominates, while in non-human primates and humans G-conjugated BAs are predominant (8). Bile fluid, which contains high millimolar concentrations of Bas, is subsequently stored in the gallbladder before it is released into the intestine upon food intake to facilitate lipid digestion. The filling of the gallbladder is regulated by FGF19, highlighting its importance in BA homeostasis (9). Within the intestine, the primary BAs CA and CDCA are converted by the intestinal microflora to the secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA), respectively, and to a minor extent to ursodeoxycholic acid (UDCA). The secondary

BAs DCA and LCA are generally more hydrophobic (due to the removal of a hydroxy group) and physiologically more potent as agonists of the BA receptors FXR or TGR5 (10-12), but also more cytotoxic compared to CA (13) due to the higher potential to disturb membranes integrity and mitochondrial function (14). The different aspects of BA toxicity in the context of liver homeostasis and drug induced liver injury is discussed and reviewed elsewhere (8). The majority of intestinal BAs are reabsorbed by enterocytes, released into the portal vein, and taken up by the liver *via* the sinusoidal membrane. Only small amounts of BAs escape this enterohepatic cycling and are excreted via the feces, accounting for approximately 10 to 20 % loss per day (15, 16).

Cholestyramine as a BA sequestrant. The BA sequestrant cholestyramine (CHO) is a strong ion exchange resin that binds BAs in the intestine and forms insoluble complexes. Intestinal binding of BAs by CHO prevents BA reabsorption and leads to enhanced BA excretion by the feces. CHO is therefore used in the clinic for management of bile acid diarrhea (17). In addition, it is also indicated to treat hypercholesterolemia due to its ability to lower serum cholesterol levels by increasing the conversion of hepatic cholesterol to BAs via indirect induction of hepatic Cyp7a1 (18). Induction of hepatic Cyp7a1 occurs secondary to CHO mediated intestinal depletion of BAs and subsequent activation of the endocrine FXR-FGF19-FGFR4- Cyp7a1 pathway (16).

Safety considerations for FGFR4 pathway inhibition. FGFR4 knockout mice have a larger BA pool with increased excretion of BAs, enhanced expression of Cyp7a1, BA-depleted gall bladders (19, 20), and hypercholesterolemia (21). Similar findings are reported for FGF15 knockout mice (mouse FGF19 ortholog) (22) and KLB knockout mice (23). Liver toxicity characterized by single cell necrosis and increased bilirubin, severe diarrhea and decreased food consumption have previously been reported in cynomologus monkeys given single or repeated doses of an anti FGF19 monoclonal antibody, and were presumably secondary to increased BAs (24).

Single or repeated doses of FGF401 in dogs led to induction of Cyp7a1 in the liver resulting in increased plasma and fecal BA content, and was associated with decreased serum cholesterol, diarrhea, and elevation of serum ALT in the absence of adverse histopathological findings in the liver, or in other organs that could be a source of ALT. In order to address if ALT perturbation could have been secondary to BA increases and its potential mitigation we conducted an investigative study in dogs co-treated with FGF401 and the BA sequestrant CHO to address this hypothesis.

Results

FGF401 induced bile acid biosynthesis, increased serum ALT and decreased cholesterol in the 4-week dog toxicity study. Repeated oral (gavage) administration of FGF401 to beagle dogs for 4 weeks at dose levels of 5, 15 and 45 mg/kg/day was overall well tolerated with only limited, dose-dependent in-life signs such as salivation, vomiting, and liquid or soft feces.

In the liver, the treatment with FGF401 resulted in a significant increase in Cyp7a1 mRNA levels (Figure 2A) which confirms blockage of the FGFR4 receptor by FGF401. The lack of a clear dose-response relationship after 4 weeks of treatment suggests full target saturation and signaling pathway activation already at the 5 mg/kg/day dose with no further enhancement at higher doses. Treatment with FGF401 at doses ≥ 5 mg/kg/day led to increased levels of C4 in plasma starting between 3 to 7 hours post-dose on day 1 (Figure 2B). The increases in plasma C4 concentrations were sustained and more pronounced after 4 weeks of treatment (Figure 2C). The changes in circulating C4 were consistent with the induction of hepatic Cyp7a1 mRNA by RT qPCR both after single (data not shown) and repeated dose administration.

Analysis of the BA profiles showed that the major BA components in control dog plasma were taurine-conjugated TCA (39%), TCDCA (25%), TDCA (21%), TLCA (4%) and unconjugated CA (7%) (Figure 3A), whereas in control dog feces, the unconjugated secondary BAs DCA (61%) and LCA (28%) were most abundant, followed by lower amounts of the unconjugated primary BAs CA (5%) and CDCA (3%) (Figure 3B).

Treatment at doses ≥ 5 mg/kg/day FGF401 for 4 weeks resulted in increases in the major BA components in plasma (Figure 3C), which was already seen after single administration at the higher doses, yet less pronounced (data not shown). Consistent with the FGF401-related increases in plasma BAs, the corresponding unconjugated primary and secondary BAs were also increased in fecal samples upon treatment (Figure 3D). Taken together, the data indicated a general increase in BA pool size upon FGF401 treatment as a consequence of increased BA biosynthesis due to FGFR4 inhibition.

Dogs treated with FGF401 showed mildly decreased total cholesterol concentrations in serum (Figure 4A, both high-density and low-density lipoprotein cholesterol) and minimally to moderately (1.5- 5.6 fold) increased serum alanine aminotransferase (ALT) activity at all doses (Figure 4B). Effects on ALT activity and cholesterol concentrations were more apparent from treatment day 8, did not become progressively more severe over time and exhibited reversibility at the end of the 4-week treatment-free phase. Minimal increases in serum aspartate aminotransferase (≤ 1.7 fold, Figure 4C) in individual animals were also noted, particularly in those with the highest levels of ALT. Despite the increase in ALT, there were no adverse histopathological findings in the liver or in any other organs. While the occurrence of liquid/soft feces and the decrease in serum cholesterol were likely the consequence of increases in BA biosynthesis, pool size and intestinal BA content (16, 25), the cause for the transient increases in ALT without histopathological correlate remained undetermined in the 4-week toxicity study.

Investigative dog study with FGF401 treatment and co-administration of cholestyramine

The investigative dog study was designed in a way to address whether the FGF401-mediated ALT increase was secondary to increases in BAs and could be prevented and reversed by co-administration of the BA sequestrant CHO. A crossover study design with different phases and washout periods was applied similarly to a clinical setting in order to increase the statistical power and identify non-, weak-, or strong responders, and follow up on the time-course of FGF401-induced effects with and without CHO intervention (Figure 5). FGF401 was administrated at a dose of 5 mg/kg/day, and CHO at a dose of 5 grams per animal approximately 2 hours after FGF401 administration, both by oral gavage. In an initial feasibility study phase the effects of CHO administration on tolerability and FGF401 exposure profile were investigated. However, CHO was well tolerated and did not compromise the absorption or change the exposure profile of FGF401 (data not shown).

Cholestyramine mitigates and reverses FGF401 mediated increases in ALT. As shown in Figure 6, the treatment with FGF401 alone caused minimal to moderate increases in serum ALT (≤ 4.3 fold), whereas co-administration of FGF401 with CHO did not result in increases in ALT levels. Furthermore, ALT levels

decreased relatively quickly to pretest values when FGF401 administration was discontinued or by adding CHO to dogs being treated with FGF401. In contrast, stopping administration of CHO and continuing FGF401 administration resulted in an ALT increasing trend.

Cholestyramine prevents FGF401 mediated increases in plasma BA by increased fecal excretion. As expected, the treatment with the BA sequestrant CHO led to increased fecal excretion of BAs as confirmed by the increased BA content in feces (Figure 7A), and decreased the BA concentrations in plasma (Figure 8). This effect was most pronounced for the hydrophobic secondary BAs DCA and LCA in feces and their corresponding taurine-conjugates TDCA and TLCA in plasma (Figure 7B). Consistent with our observation in the 4-week dog study, treatment with FGF401 alone resulted in increases in plasma BAs, driven both by primary and secondary BAs (Figure 8). When FGF401 was co-administered with CHO, plasma BA concentrations remained comparable to untreated controls or were even decreased (Figure 8A), this effect being more pronounced for the secondary BAs TDCA and TLCA (Figure 8C). When FGF401 administration was discontinued or when CHO was co-administered to dogs being treated with FGF401, increased plasma BA levels decreased relatively quickly to or below untreated control values. Thus, the FGF401-mediated increases in plasma BAs could be mitigated and reversed by co-treatment with CHO, overall in a similar way as CHO was able to mitigate and reverse FGF401-mediated increases in serum ALT.

Potential association between hydrophobic BAs and ALT increase. Correlation analysis (TIBCO Spotfire Linear Regression) at peak ALT levels revealed that the FGF401-mediated increase in serum ALT correlated best with plasma BAs of high hydrophobicity, such as TLCA (study day 14: $r^2 = 0.79$, $p<0.001$; study day 38: $r^2 = 0.83$, $p<0.0001$), TDCA (study day 14: $r^2 = 0.82$, $p<0.0001$), the sum of these secondary BAs (study day 14: $r^2 = 0.82$, $p<0.0001$; study day 38: $r^2 = 0.57$, $p<0.01$), and the ratio of hydrophobic to polar BAs (sum of TLCA, TDCA, TCDCA divided by sum of TCA, CA; study day 14: $r^2 = 0.72$, $p<0.001$; study day 38: $r^2 = 0.73$, $p<0.001$). Consistently, a principal component analysis over all data points of the study also showed that plasma TLCA and TDCA showed a higher degree of correlation with ALT elevation

as compared to plasma TCDCA and TCA. These data therefore suggest a potential prominent role of hydrophobic, particularly secondary BAs in the mechanism of ALT elevation, and thus of the hydrophobicity of the BA pool. The secondary BAs are formed in the intestine by the microbiome at high abundance, and were also the ones most affected by the treatment with CHO.

Effect of FGF401 and cholestyramine on cholesterol and C4.

As shown in Figure 9A, treatment with FGF401 or CHO resulted in decreased serum total cholesterol concentrations, which quickly reversed to pretest levels upon treatment discontinuation. Consistent with our data from the 4-week toxicity study, treatment of dogs with FGF401 increased plasma C4 concentrations, which became apparent after the first administration and appeared to plateau after 6 to 7 days of repeated daily dosing (Figure 9B). As expected, when CHO was administrated alone in the first phase of the study, plasma C4 concentrations increased as well, indicating induction of hepatic Cyp7a1 activity by the CHO treatment. When FGF401 was co-administered with CHO, plasma C4 concentrations increased further compared to FGF401 or CHO administration alone, which resulted in an additional elevation of C4 levels.

Analysis of miR-122 in plasma as marker for hepatocellular injury. To investigate whether the liver might be the source of FGF401 mediated ALT elevation despite the lack of observable histopathological changes, exploratory miR-122 analysis was conducted in plasma as a biomarker for hepatic injury. Overall, circulating miR-122 levels were highly variable over the course of the study. The treatment with FGF401 did not result in a consistent change in plasma miR-122 with the exception of two individual animals, which showed minimal to moderate increases in miR-122 when treated with FGF401 alone (study days 25 to 38), followed by a trend towards a decrease after co-treatment with CHO (Figure 10). The highest level of miR-122 and ALT was seen in one out of these two animals, suggesting a hepatocellular origin of the ALT increase in that dog. Otherwise, there was no clear correlation between FGF401-induced ALT elevation

and changes in miR-122, although miR-122 appeared slightly more elevated in FGF401 treated animals than with FGF401-CHO combination.

Discussion

Inhibition of the FGF19/FGFR4/KLB pathway is a promising target to treat patients with HCC, but is generally associated with modulation of BA homeostasis and increased BA pool size (4). Indeed, we showed that blockage of the FGFR4 receptor by the antagonist FGF401 led to upregulation of Cyp7a1 mRNA in the liver, increased BA biosynthesis and BA content in plasma and feces in mice and dogs. The treatment with FGF401 was also associated with transient, minimal to mild increases in serum ALT without observable adverse histopathological findings in the liver or in any other tissues, making the cause of the ALT elevation unclear. We therefore sought to determine whether the FGF401-mediated increases in ALT were secondary to BA increases and conducted an investigative study in dogs with FGF401 treatment and co-administration of the BA sequestrant CHO. CHO administration itself did not affect levels of ALT in dogs in the investigative study, which was tested in the initial phase of the study in view of some clinical reports in healthy volunteers associating CHO treatment with increases in ALT (26-28). No treatment-related effects on mortality/moribundity, clinical signs, body weight or food consumption parameters were noted at any dose or combination of doses with FGF401 and CHO. The lower levels of serum total cholesterol with FGF401 have also been noted in the 4-week dog study with daily dosing of FGF401. In addition, decreases in serum total cholesterol were observed in dogs treated with CHO alone, which is an anticipated effect of intestinal sequestration of BAs (18). In both cases, the mechanism behind the reduction in serum cholesterol is likely increased BA synthesis in the liver, which leads to increased consumption and subsequent depletion of hepatic cholesterol and in turn to increased cholesterol uptake from the circulation (16). Consistently, plasma C4 levels were increased in dogs treated with FGF401 both in the 4-week and in the investigative dog study, indicating FGFR4 antagonism with subsequent induction of hepatic Cyp7a1 and BA biosynthesis. Thus, circulating C4 appears to be a suitable pharmacodynamic marker of FGFR4 inhibition. However, CHO administration alone induced increases in plasma C4 as well, indicating hepatic induction of Cyp7a1 secondary to intestinal BA adsorption to the resin and increased excretion, which prevents activation of the endocrine FXR/FGF19/FGFR4/KLB/Cyp7a1 pathway (16). Consequently, C4

needs to be considered carefully ***[interpreted with caution?]*** as a surrogate marker for the pharmacodynamic response when FGF401 is co-administered with CHO in patients.

FGF401-mediated increases in serum ALT were directly modified by co-administration of CHO. Indeed, co-administration of FGF401 and CHO prevented an increase in ALT, whereas administration of FGF401 alone caused increased ALT levels up to approximately 4-fold in single animals compared to baseline. Following the same time-course, FGF401 administration alone increased plasma BAs concentrations due to increased biosynthesis paralleling the elevation in ALT, which was prevented and reversed by co-administration with CHO. CHO binds BAs within the intestine, prevents its absorption and leads to increased fecal excretion as shown by the increased BA content in feces, which in turn decreased BAs in the circulation and reversed FGF401-related increases. Our results therefore demonstrate that lowering the amount of free BAs in the intestine by CHO can prevent both FGF401 mediated increases in circulating BAs and ALT. In the ongoing clinical trial with FGF401 in HCC patients in whom CHO is used for management of diarrhea, we have observed a trend towards lower levels of serum aminotransferases in patients given CHO. These clinical data, while early and limited, together with the results of our investigative dog study, suggest that CHO may have the potential to mitigate transaminase increases in the clinic in instances where BAs may be involved in ALT elevation.

By investigating the potential associations between individual BA components and changes in ALT throughout the course of the investigative study, it appeared that the secondary BAs TDCA, TLCA, and thus the increased hydrophobicity of the BA pool, were strongly associated with increases in ALT. Secondary BAs are formed in the intestine by the microbiome (29), are generally more hydrophobic compared to primary BAs, and were strongly reduced in the circulation by the treatment with CHO. These hydrophobic BAs are also the ones with higher potency on BA receptors such as FXR or TGR5 (10-12), and exhibit higher cytotoxicity compared to the more polar ones (30) due to a higher potential to disturb membrane integrity and mitochondrial function (14). Thus, a mechanistic link between the FGF401-mediated elevation in ALT and increased BAs, particularly of those with high hydrophobicity, appears

plausible and could involve various mechanisms including direct BA induced hepatocyte toxicity. However, a direct hepatotoxic effect of BAs on the liver as a potential cause for FGF401-mediated ALT elevation was not supported by our data based on the absence of histopathological observations in the 4-week dog toxicity study.

To further investigate the origin of ALT elevations in the non-terminal investigative dog study, analysis of circulating miR-122 as an exploratory biomarker for hepatic injury was conducted (31). Although miR-122 appeared slightly higher in FGF401 treated animals than with the FGF401-CHO combination in some phases of the study, there was no consistent change and the results overall did not clearly correlate with the observed FGF401-related increases in ALT. Only one animal showed a minimal to mild increase in plasma miR-122 on FGF401 treatment that was associated with increased ALT levels, which might point towards a hepatic origin of the ALT in that specific animal. Interestingly, the same animal had among the highest levels of plasma TLCA upon FGF401 treatment, which is a highly hepatotoxic BA in experimental animals (29) and may have affected the membrane integrity and caused leakage from hepatocytes. In humans, TLCA and GLCA are efficiently sulfated at C-3 position which prevents their reabsorption from the intestine and leads to excretion in feces (29).

A number of publications show increased serum liver enzyme levels in the absence of liver injury, and various potential mechanism have been investigated (32, 33). To address whether FGFR4 pathway inhibition led to upregulation of the Gpt gene coding for ALT in the liver, we performed gene expression analysis by RT qPCR in the 4-week dog study but did not observe any FGF401-related changes.

Increased serum enzyme levels without signs of hepatic or skeletal muscle injury can be due to reduced Kupffer cell-mediated clearance of serum enzymes (34, 35). Kupffer cells are resident macrophages lining the hepatic sinusoids that clear several serum enzymes by phagocytosis and endocytosis, including creatinine kinase and aminotransferases. It was shown that reducing the number of Kupffer cells, *e.g.* by neutralizing macrophage colony-stimulating factor via an antibody, led to increased serum enzymes levels

without evidence of tissue injury. Interestingly, Kupffer cells express the BA receptor TGR5 and are functionally suppressed in the presence of hydrophobic BAs, which transiently attenuate their phagocytotic activity (36, 37). The potency of BAs on the TGR5 receptor is ranked in the order LCA ≥ DCA > CDCA > CA, with a stronger activation potential for the corresponding T-conjugated BAs TLCA ≥ TDCA > TCDCA > TCA (12). Notably, the results of our investigative dog study with FGF401 and co-administration with CHO indicates an association between FGF401-mediated increases in serum ALT activity and increases in BAs, predominantly with the secondary BAs of high hydrophobicity such as TLCA and TDCA. Thus, it could be speculated that the transient increase in serum ALT activity without signs of liver injury might have been caused by a transient reduction of phagocytotic Kupffer cell activity due to increased activation of the TGR5 receptor by TLCA and TDCA, leading to decreased clearance. Such a mechanism would be consistent with the observed association between increases in circulating ALT and the secondary BAs TLCA, TDCA in dogs after treatment with FGF401 and the possibility to mitigate this effect by CHO.

Liver sinusoidal endothelial cells (LSEC) are also involved in the clearance of serum enzymes and immunoglobulins by endocytotic uptake (38-40) and are responsive towards specific BAs by expressing the TGR5 receptor (41). Whether TGR5 and thus BAs are directly involved in the regulation of the endocytotic activity of LSECs has not been investigated by our group. For LSECs, differences in the clearance kinetic and specificities for serum enzymes have been reported (38, 40). This could explain why different serum aminotransferases are differently affected by changes in BAs concentrations. Considering their localization within the sinusoid, both LSECs and Kupffer cells are exposed to high concentrations of secondary, hydrophobic BAs from the portal blood after intestinal reabsorption, and thus with high local abundance of their primary TGR5 ligands at their cell surface. However, further investigations are necessary to characterize such a mechanism, which could also co-occur with minimally increased hepatocyte leakage due to the presence of hydrophobic BAs and undetected in histopathology.

In conclusion our data showed that co-administration of the BA sequestrant cholestyramine prevented and reversed FGF401-mediated increases in serum ALT and may have utility to mitigate serum transaminase elevations in the clinic in instances of BA involvement, independent of its use for diarrhea management.

Methods

Animals and treatment. For the 4-week toxicity study, male and female beagle dogs obtained from Marshall BKU Ltd (Grimston, UK) were approximately 10 months to 12 months of age, and weighed 5.7 to 10.2 kilograms at the initiation of dosing. In this study, FGF401 was administrated as a solution of 100 mM citrate buffer, pH 2.5, by oral gavage to groups of 3 or 5 dogs per sex at dosages of 0 (vehicle), 5, 15 and 45 mg/kg/day using a dosage volume of 5 mL/kg. After cessation of treatment, two animals per sex in the control and high-dose group were maintained on the study for a 4-week recovery period.

For the non-terminal investigative study, male beagle dogs obtained from Marshall BioResources (North Rose, NY), were approximately 7 months to 3 years of age, and weighed 7.2 to 11.1 kilograms at the initiation of dosing. FGF401 and cholestyramine (CHO; Questran Powder [Cholestyramine for Oral Suspension, USP]; NDC no. 49884-09366-66; lot no. 26079601; PAR Pharmaceutical Companies, Inc., Spring Valley, NY, USA) were each administered by oral gavage to beagle dogs as a formulation in their respective vehicles [FGF401, in 100 mM citrate buffer, pH 2.5; CHO, in drinking water]. The dose volume for the FGF401 formulation was 5 mL/kg. For CHO, the dosing aliquot was administered in 75 mL drinking water. For the investigative dog study, an initial feasibility study phase was conducted to investigate potential effects of CHO on the tolerability and exposure profile of FGF401. For this purpose, 6 stock animals were administered FGF401 at a single dose of 5 mg/kg, followed by dosing with vehicle to CHO (first 3 animals) or CHO at 5 g/dog, corresponding to 11.25 g Questran powder (last 3 animals), approximately 2 hours later. Food was presented after the second dosing event. Blood was collected for determination of FGF401 plasma concentrations at approximately 0.5, 1, 3, 7 and 24 hours after FGF401 administration. Animals were returned to colony on day 2. For the main study phase of the investigative study, a crossover design was applied similarly to a clinical setting in order to identify non-, weak or strong responders to FGF401 treatment and to follow-up on the time-course of FGF401-induced effects after CHO intervention with different biomarkers in relation to systemic exposure on an individual basis. Two

groups of 6 naïve animals were used (Group 1 with animal nos. 1006 to 1006 and Group 2 with animal nos. 2001 to 2006). The main study phase started with dosing of either vehicle or CHO at 5 g/animal for one week (phase I). FGF401 dosing at 5 mg/kg/day was initiated on study day 8 (phase II), followed approximately 2 hours later by a second administration of either vehicle or CHO (5g/animal) at each day. Food was presented to the animals after the second daily dosing event. Animals were not dosed on study days 15-24 (washout/recovery period, phase III). Dosing with FGF401/CHO was restarted on study day 25 (phase IV) and the dose groups crossed over on study days 39-44 (phase V), with the last day of dosing on study day 44. The animals were returned to colony on study day 53. The administration schedule including cross-over design is shown in Figure 11.

Clinical observations, body weight and food consumption. Daily clinical observations, once- or twice-weekly body weights, and daily food consumption determinations were performed.

Clinical pathology: In the 4-week dog toxicity study, blood samples for clinical chemistry (*e.g.* serum ALT, AST, total cholesterol) were collected once before initiation of dosing (pretest), on treatment days 2, 8, 15, 22, and 26 approximately 24 hours postdose, and on days 3, 8, 15, and 26 of the treatment-free recovery period. In the investigative dog study, blood samples for clinical pathology assessments were collected once during pretest (hematology, clinical chemistry, and coagulation) for both the feasibility study phase and the main study phase. For the main study phase, blood sampling for clinical chemistry was conducted at approximately 3 hours postdose (post vehicle, CHO or FGF401 dosing, as appropriate, or at similar time of the day during washout periods) on study days 1, 3, 7, 8, 11, 14, 15, 18, 21, 23, 25, 28, 31, 35, 38, 42, and 45.

Toxicokinetics: Plasma samples for toxicokinetic (TK) analysis were collected from all animals approximately 0.5, 1, 3, 7, and 24 hours post FGF401 dosing on study days 1 and 26 of the 4-week toxicity study, as well as in the single-dose feasibility phase and in the main study phase on study days 8, 14, 25, 39 and 44 of the investigative study.

C4/BA profiling by LC-MS. Plasma samples for BA profiling and C4 analysis were collected on study days 1 and 26 concurrent to TK sample collection in the 4-week toxicity study, and in the investigative study during the main study phase concurrent with clinical chemistry and TK. Feces samples for BA analysis were collected at the end of treatment week 4 in the 4-week study, and in the investigative study at pretest and on study days 7 and 49 of the main study phase. BA profiles and C4 analyses were carried out on an Agilent 1290 Infinity ultra high-performance liquid chromatography (UPLC) system interfaced to an ABSciex API4000 triple-quadrupole tandem mass spectrometer (LC-MS/MS) operated in positive electrospray ionization mode. All data were acquired and analyzed using Analyst 6.1 data processing software. Reference standards and deuterated internal standards (IS) for calibration and quantification were obtained from Sigma-Aldrich (CA, CDCA, DCA, LCA, TCDCA, d4-CA, d4-CDCA, d4-LCA), Steraloids (TLCA, d4-DCA), Calbiochem (TCA, TDCA), C/D/N Isotopes (d4-UDCA, d4-GCA, d4-GCDCA, d4-GUDCA) and Toronto Research Chemicals (C4 and d7-C4). All other chemicals and reagents were purchase from Sigma-Aldrich and were of the highest purity available. Stock solutions for BA and IS were prepared in methanol (MeOH, 10 mM), combined and diluted to obtain mixed working solutions for BAs and IS at concentrations of 100 µM, each. Calibration standards for analysis of plasma samples were prepared by spiking defined volumes of serially diluted BA working solutions to 50 µL of aqueous 20% bovine serum albumin solution (BSA, fatty acid free). Quality control (QC) samples were prepared in control mouse, rat and dog plasma containing a low, mid and high concentration of each analyte. Plasma samples (50 µL), QCs and calibration standards were processed by addition of 50 µL of mixed IS working solution and 450 µL acetonitrile (ACN) and thoroughly mixed. Samples were centrifuged at >14'000 × relative centrifugation force (rcf) for 15 min at 4ºC, the supernatants were transferred into new tubes and evaporated to dryness in a vacuum centrifuge. After reconstitution in 60 µL MeOH/water 1:1 (v/v) containing 0.1% formic acid (FA), 5 µL were injected into the LC-MS/MS system for analysis. For BA analysis in feces, a multi-step extraction procedure was applied using solvents with different polarities and acidification according to a protocol by Manes et al. (42) with modifications, to enable BA extraction both from naïve and CHO containing feces. An aliquot of approximately 2 g of

fecal sample was accurately weighed in a glass flask, diluted 1:10 (w/w) in water, mixed and allowed to soak/swell at 4°C. The samples were thoroughly mixed to obtain a homogenous slurry. Two mL of the homogenous slurry (corresponds to 200 mg fecal samples) were transferred into a weighed 15 mL plastic tube and centrifuged at 5'000 x rcf for 10 min. The supernatant was removed, transferred into a new tube, and later combined with the extracts of the 4 subsequent extraction steps of the residual pellet, which were as follows: 4 mL of MeOH, 4 mL of ACN and 2 x 4 mL of acidified ethanol containing 0.5 N hydrochloric acid (HCl). An aliquot (900 µL) of the combined extracts was transferred into a new tube, spiked with IS, mixed, and evaporated to dryness in a vacuum centrifuge. After reconstitution in 500 µL MeOH/water 1:1 (v/v) containing 0.1% FA, 5 µL were injected into the LC-MS/MS system for analysis. Calibration standards were prepared by spiking defined volumes of serially diluted BA working solutions to a mixture of MeOH:ACN:water 4:4:1 to reflect a similar solvent constitution as in the samples, evaporated to dryness in a vacuum centrifuge and reconstituted in 500 µL methanol/water 1:1 (v/v) containing 0.1% FA for LC-MS/MS analysis. The dry weight of the fecal samples was determined by drying the residual pellet after extraction to constant weight.

microRNA analysis (miR-122). Plasma samples for microRNA analysis were collected from all animals during the main study phase of the investigative study time-matched with clinical chemistry sample collection. MicroRNA analysis was performed according to a protocol published by Thompson and coworkers (43). Small RNA was extracted from 50 µl of plasma by using the miRNeasy/MinElute kit and following the instructions of the manufacturer (Qiagen). Elution of small RNA was conducted in 14 µl of nucleic acid-free water. 3.2 µl of eluted small RNA was reverse transcribed into cDNA by using the Megaplex RT primer human pool A and B and the Taqman MicorRNA Reverse Transcription kit (Applied Biosystems / Life Technologies, reference 4366596), following the instructions of the manufacturer. 2.5 µl of cDNA was preamplified by using the Megaplex Preamp primer human poolA and B (Applied Biosystems / Life Technologies, reference 4399201 and 4399233) and the Taqman PreAmp Master mix (Applied

Biosystems / Life Technologies, reference 4391128), following the instructions of the manufacturer. The microRNA miR-122 was amplified from preamplified cDNA by using a commercially available validated Taqman assay (reference 002245) diluted in the Taqman MasterMixII (Applied Biosystems / Life Technologies), following the instructions of the manufacturer. The relative quantification of plasmatic microRNA levels was performed using the standard curve method to generate expression values as number of molecules.

Analysis of mRNA expression in liver. Liver samples for mRNA levels analysis were collected at scheduled necropsy at approximately 4 hours post last FGF401 dose administration in the 4-week toxicity study and immediately snap frozen in liquid nitrogen. Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol, Invitrogen Life Technologies) from each frozen tissue section and the total RNA was purified on an affinity resin (Rneasy, Qiagen) according to the manufacturer's protocol. Total RNA was quantified by the absorbance at λ = 260 nm (A260nm), and the purity was estimated by the ratio A260nm/A280nm. RNA integrity was controlled by running a Bioanalyzer profile (Agilent). RNA was stored at -80°C until analysis. 300 ng of total RNA was reverse transcribed into cDNA by using the High Capacity cDNA Archive Kit according to the manufacturer protocol (Applied Biosystems). PCR was performed in triplicate from 6 ng of cDNA by using the SYBR Green Real-time PCR master mix following the instructions of the manufacturer (Applied Biosystems). Quantification of gene expression changes was performed by using the standard curve method. 18S RNA values were used for normalization and data from technical triplicate were averaged. Sybrgreen assays were either purchased from Qiagen (18S #QT00199367, Gpt #QT01353142) or designed and validated in house as for Cyp7a1 (Cyp7a1 forward primer: CAGGCCTCCCCATTCATGT, Cyp7a1 reverse primer: AAGCCCTCTGCCAGCTTTTC).

Statistics.

GraphPad Prism 7.03 (GraphPad Software) was used for statistical analysis. Data are expressed as mean ± SEM. Differences among multiple groups were examined using one-way ANOVA with Dunnett's multiple comparison test versus control group. Differences between two groups were evaluated using an

unpaired, 2-tailed Student's *t* test. *P* values below 0.05 were considered significant. TIBCO Spotfire was used for correlation analysis by linear regression, principal component analysis (PCA) was performed with prcomp function in R(version 3.4.1).

Study approval and animal welfare.

These studies were performed in accordance to the respective national animal welfare regulations and approved animal study licenses. The 4-week toxicity dog study was conducted in accordance with the requirements of the UK law (Animals (Scientific Procedures) Act 1986). The non-terminal investigative dog study was conducted in accordance with the United States Department of Agriculture Animal Welfare Regulations and with the Novartis Animal Care and Use Committee-approved protocol no. TX 4039.

Words: 8760

Author contributions

HS: Design of research study, data analysis and interpretation, writing or contributing to manuscript
AW: Design of research study, data analysis and interpretation, writing or contributing to manuscript
JM: Design of research study, data analysis and interpretation, writing or contributing to manuscript
KW: Design of research study, data analysis and interpretation, writing or contributing to manuscript
PC: Data analysis and interpretation, writing or contributing to manuscript
MS: Data acquisition
AF: Data acquisition
ML: Data acquisition
MP: Data analysis
CE: Data acquisition, analysis and interpretation
CHT: Design of research study
ES: Data acquisition, analysis and interpretation
TR: Design of research study (study director)
LM: Design of research study (Clinican)
GKU: Data analysis
FP: Writing or contributing to manuscript
SDC: Writing or contributing to manuscript
AW: Writing or contributing to manuscript
DGP: Writing or contributing to manuscript
RF: Writing or contributing to manuscript
BK: Writing or contributing to manuscript
JKA: Design of research study, data analysis and interpretation, writing or contributing to manuscript

Acknowledgments

Figures

Figure 1: Schematic overview of the FGF19/FGFR4/KLB regulated hepatic BA biosynthesis, conjugation, transport and enterohepatic cycling. Binding of FGF19 to the FGFR4/KLB receptor complex in the liver results in repression of Cy7a1 transcription, the rate limiting step in BA biosynthesis. Intestinal FGF19 expression in enterocytes is regulated by FXR, a nuclear transcription factor which is activated by intestinally reabsorbed BAs. By this feedback loop, BAs regulate their own biosynthesis. In contrast, inhibition of FGFR4 by FGF401 leads to transcriptional activation of Cyp7a1 in the liver and thus to BA biosynthesis. Hepatic Cyp7a1 activity can indirectly be monitored in a non-invasive way by the blood marker C4, which is an intermediate in BA biosynthesis and used as surrogate PD readout for FGFR4 inhibition in the present dog studies.

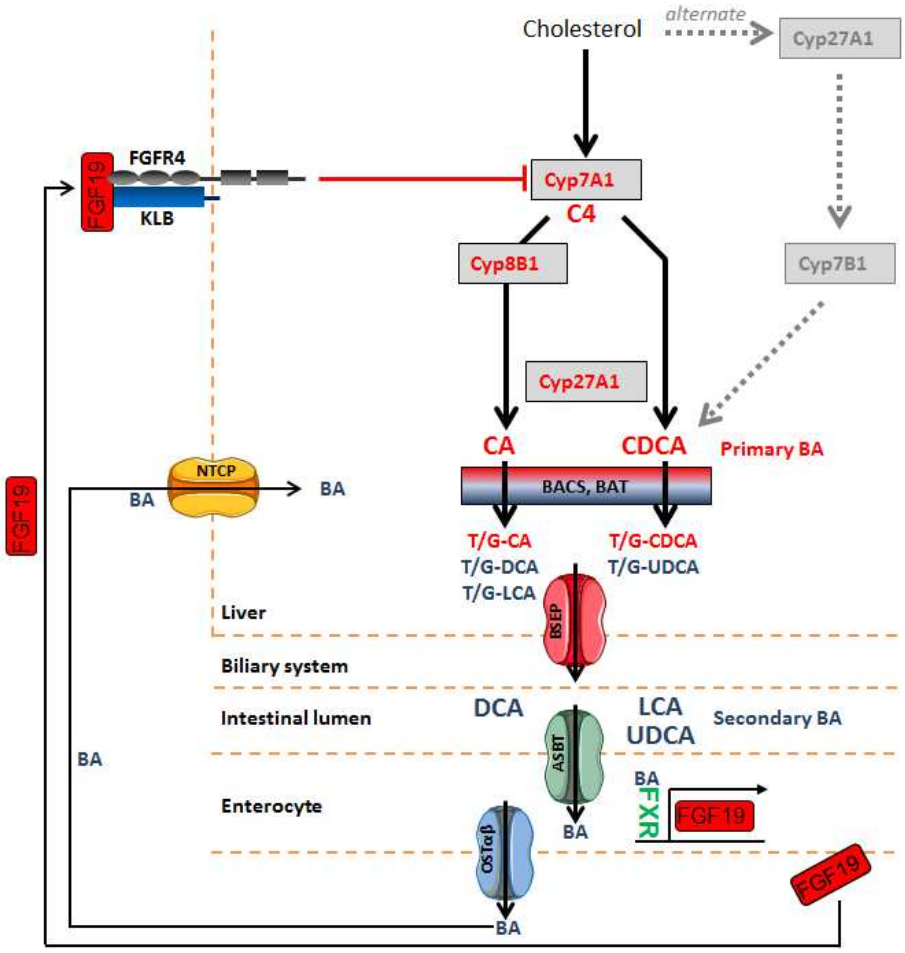


Figure 2: PD of FGFR4 inhibition in the 4-week FGF401 dog study. Dogs were treated by oral gavage with the FGFR4 inhibitor FGF401 for 4 weeks at doses of 5, 15 and 45 mg/kg/day or with vehicle. **(A)** Upregulation of Cyp7a1 mRNA transcript confirms blockage of FGFR4 by FGF401; N = 6 in each group. **(B, C)** Circulating 7α-hydroxy-4-cholesten-3-one (C4), a marker for hepatic Cyp7a1 activity and included as surrogate PD readout for FGFR4 inhibition, is increased in plasma after a single dose **(B)** and repeated daily dosing of FGF401 for 4 weeks (C), confirming antagonism on FGFR4. Area under the curve ($AUC_{0\text{-}24h}$) values were calculated for C4 based on individual plasma concentrations determined in samples collected 0.5, 1, 3, 7, and 24 hours post dose. N = 10 for vehicle control and 45 mg/kg/day groups, n = 6 for 5 and 15 mg/kg/day groups. **(A, B, and C)** Mean ± SEM are shown. *P < 0.05, ** < 0.01, and *** < 0.001, *** < 0.0001 by one-way ANOVA with Dunnett's multiple comparison test versus control group.

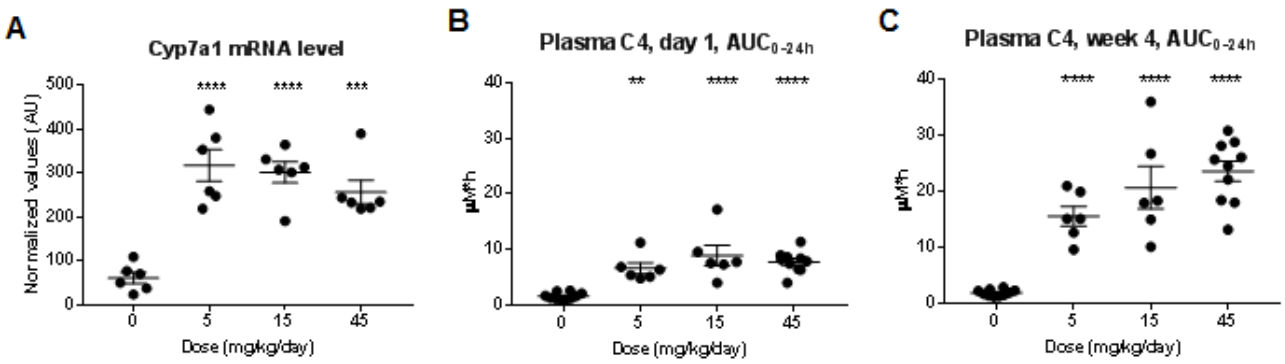
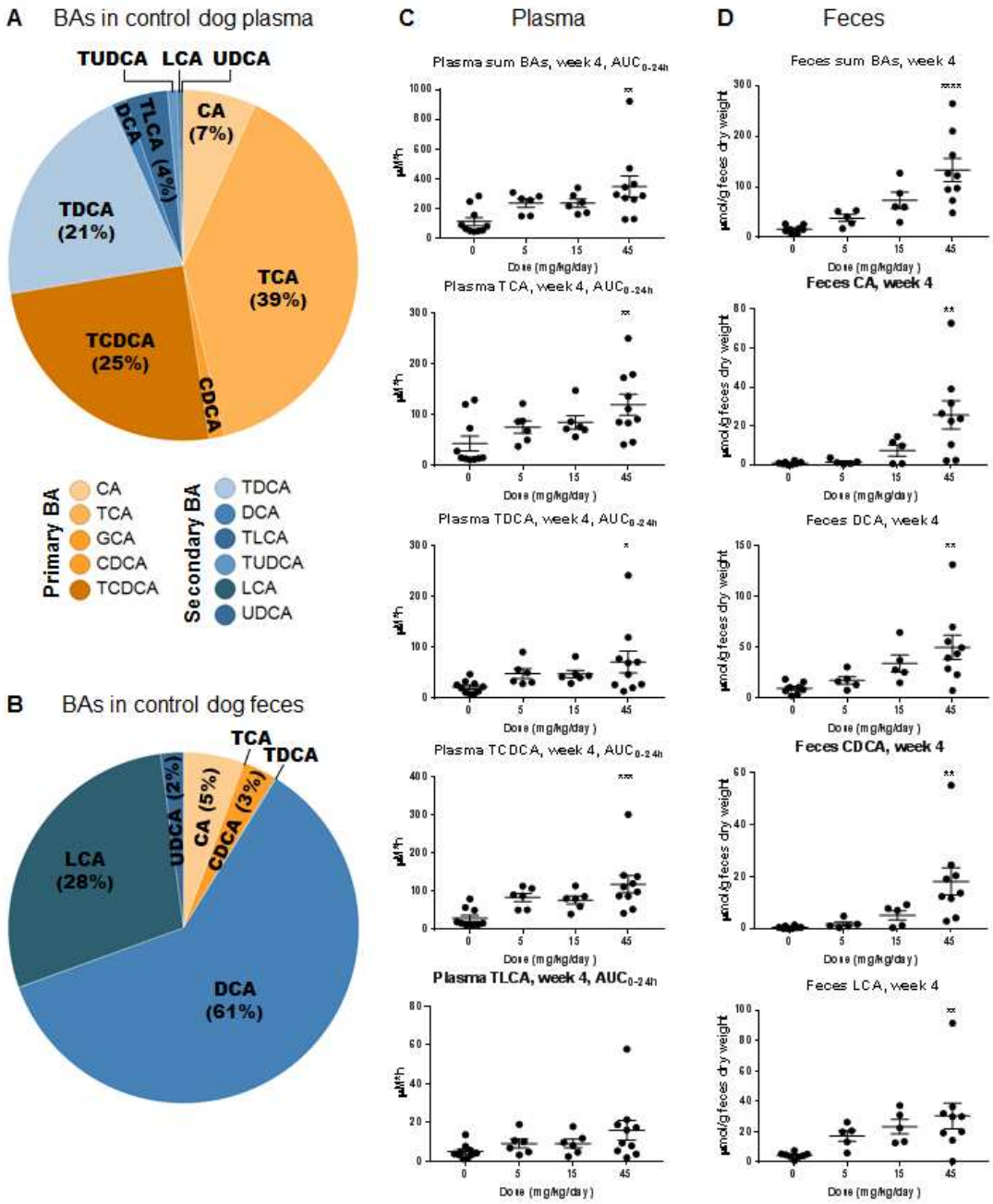


Figure 3: Bile acids in plasma and feces in the 4-week FGF401 dog study. **(A, B)** Relative BA composition in plasma (A) and feces (B) of control male and female dogs. **(C, D)** Treatment with FGF401 for 4 weeks at doses of 5, 15 and 45 mg/kg/day resulted in increases in BA components in **(C)** plasma (TCA, TDCA, TCDCA, TLCA; n = 10 for vehicle control and 45 mg/kg/day groups, n = 6 for 5 and 15 mg/kg/day groups.) and their corresponding unconjugated BAs in **(D)** feces (CA, DCA, CDCA, LCA; n = 8 for vehicle control, n = 5 for 5 and 15 mg/kg/day, and n = 9 for 45 mg/kg/day groups), respectively. **(C)** Area under the curve ($AUC_{0\text{-}24h}$) values were calculated based on individual plasma BA concentrations determined in samples collected 0.5, 1, 3, 7, and 24 hours post dose. **(C, D)** Mean ± SEM are shown. *P < 0.05, ** < 0.01, and *** < 0.001, *** < 0.0001 by one-way ANOVA with Dunnett's multiple comparison test versus control group.



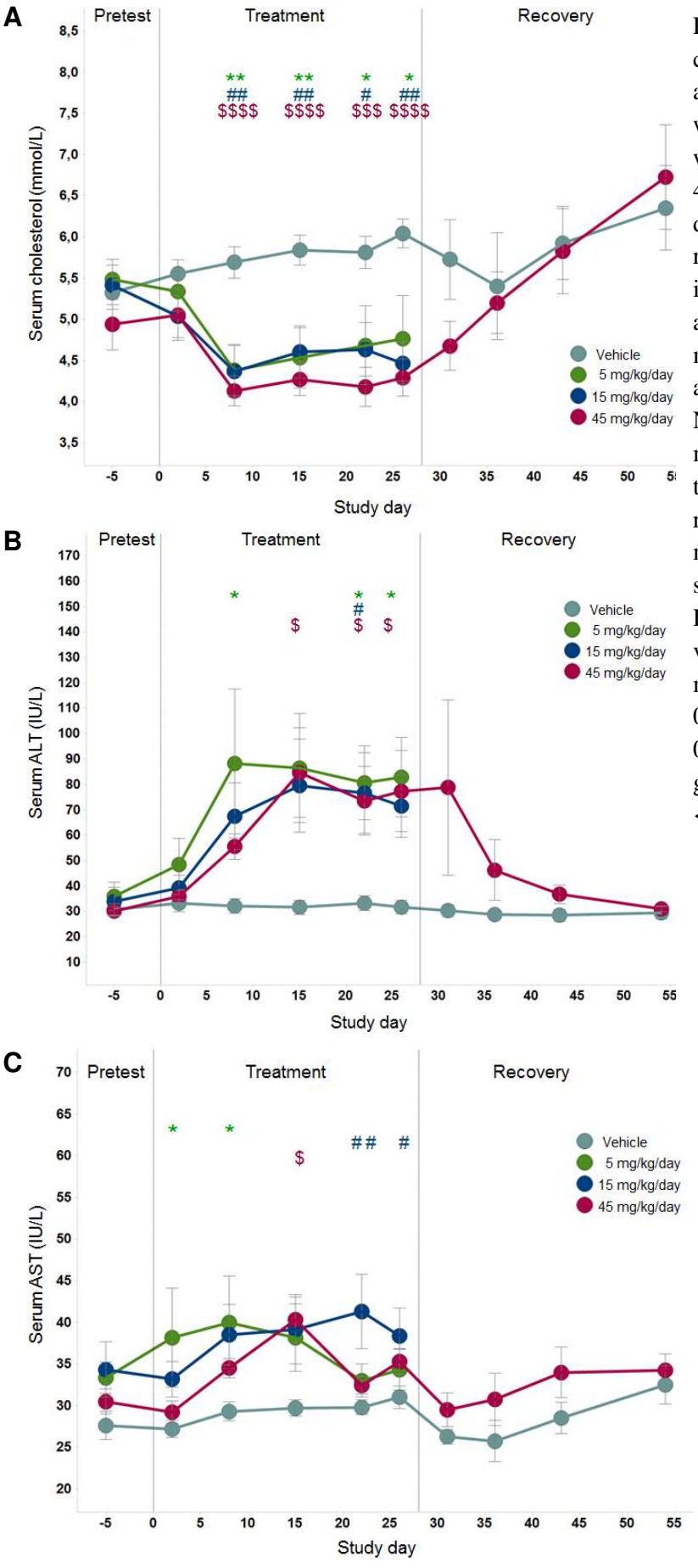


Figure 4: Changes in total cholesterol, serum alanine and aspartate aminotransferases in the 4-week FGF401 dog study. Treatment with FGF401 at doses of 5, 15, and 45 mg/kg/day resulted in **(A)** decreases in total cholesterol, **(B)** reversible, minimal to moderate increases in serum alanine aminotransferase activity, and **(C)** minimal increases in serum aspartate aminotransferase activity. **(A, B, C)** N = 10 for vehicle control and 45 mg/kg/day groups during the treatment period, and n = 2 during recovery period; n = 6 for 5 and 15 mg/kg/day groups. Mean ± SEM are shown. One-way ANOVA with Dunnett's multiple comparison test versus vehicle control group. 5 mg/kg/day group: * $p < 0.05$, ** $p < 0.01$; 15 mg/kg/day group: # $p < 0.05$, ## $p < 0.01$; 45 mg/kg/day group: $ $p < 0.05$, $$ $p < 0.01$, $$$ $p < 0.001$, $$$$ $p < 0.0001$.

Figure 5: Investigative dog study with FGF401 and cholestyramine. Crossover study design of the investigative dog study with 5 mg/kg/day FGF401 administration and co-administration of the BA sequestrant cholestyramine (CHO, 5 g per animal). In phase I, the effect of CHO on BAs and clinical chemistry parameters was assessed. In study phases II and V, group 1 animals were daily treated with FGF401 (vehicle to CHO was water), while group 2 animals were daily treated with FGF401 and CHO (approximately 2 hours post FGF401 administration). Phase IV consisted of a treatment crossover, phase III and VI were washout/recovery phases without any treatment. N = 6 per group.

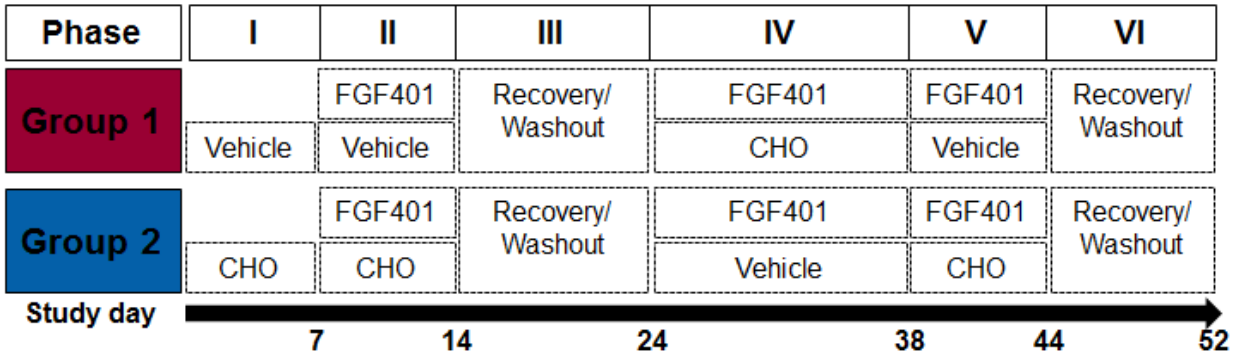


Figure 6: Changes in ALT in investigative dog study with FGF401 and cholestyramine. Treatment with CHO did not affect serum ALT levels (phase I study days 1 to 7). Treatment with 5 mg/kg/day FGF401 alone resulted in minimal to moderate increases in serum ALT activity (group 1: phase II study days 8 to 14 and phase V study days 39 to 44; group 2: phase IV study days 25 to 38), which could be mitigated and reversed by co-administration with the BA sequestrant CHO (group 2: phase II study days 8 to 14 and phase V study days 39 to 44; group 1: phase IV study days 25 to 38). Discontinuation of FGF401 treatment (recovery/washout phases III study days 15 to 24 and phase VI study day 45 onwards) or administering CHO on FGF401 (group 2: crossover from phase IV to phase V) rapidly decreased ALT levels to pretest control values. N = 6 per group. Mean ± SEM are shown.

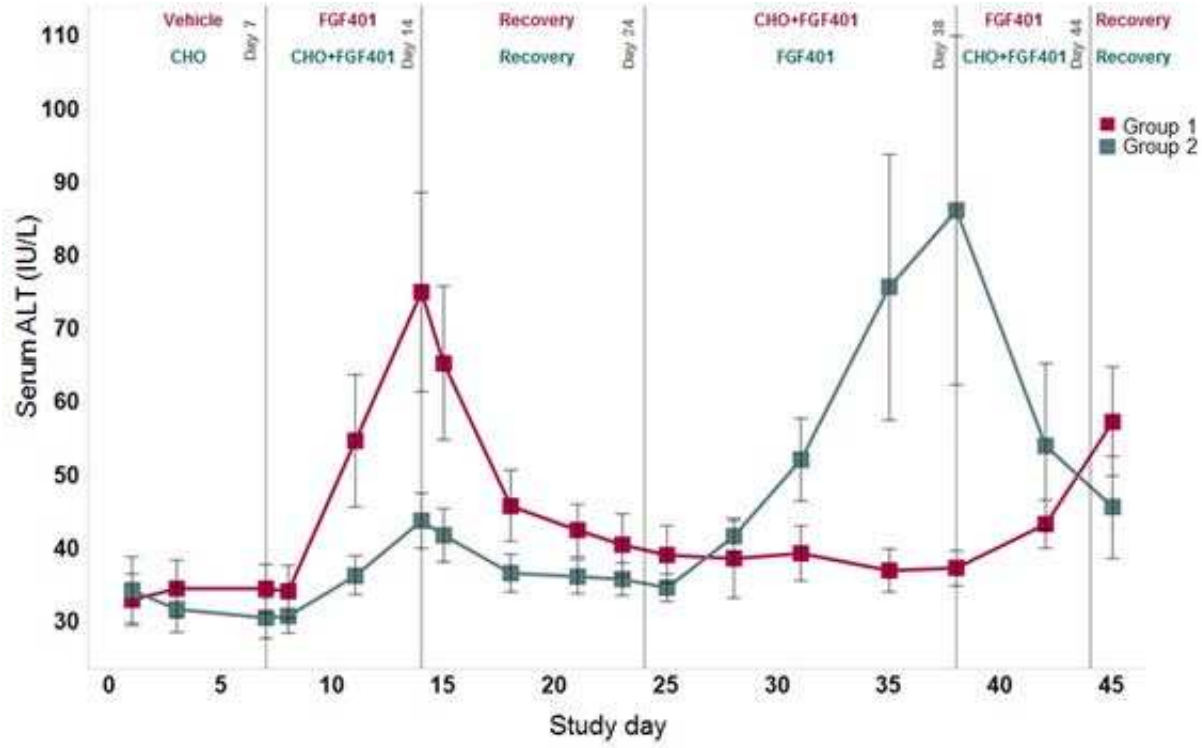
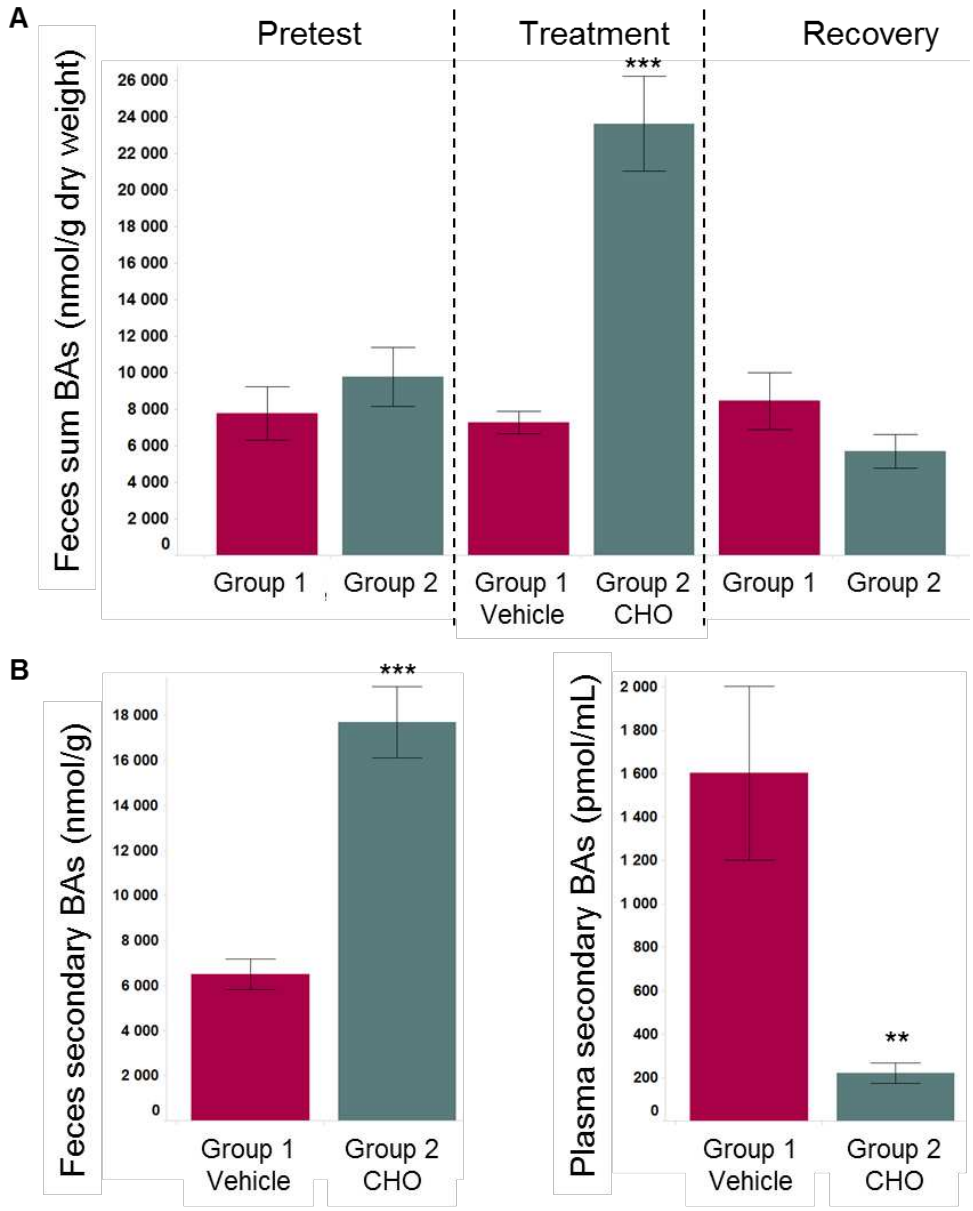


Figure 7: Effect of the BA sequestrant CHO on BAs in feces and plasma of dogs. (A) Administration of 5 grams of CHO to dogs by oral gavage led to increased fecal excretion of BAs as determined by BA content in fecal samples. (B) Increased fecal excretion of BAs was associated with decreased BA concentrations in the plasma and most pronounced for the secondary BAs DCA, LCA in feces and their corresponding conjugates TDCA, TLCA in plasma. N = 6 per group. Mean ± SEM are shown. *P < 0.05, ** < 0.01, and *** < 0.001, by students *t* test group 1 versus group 2



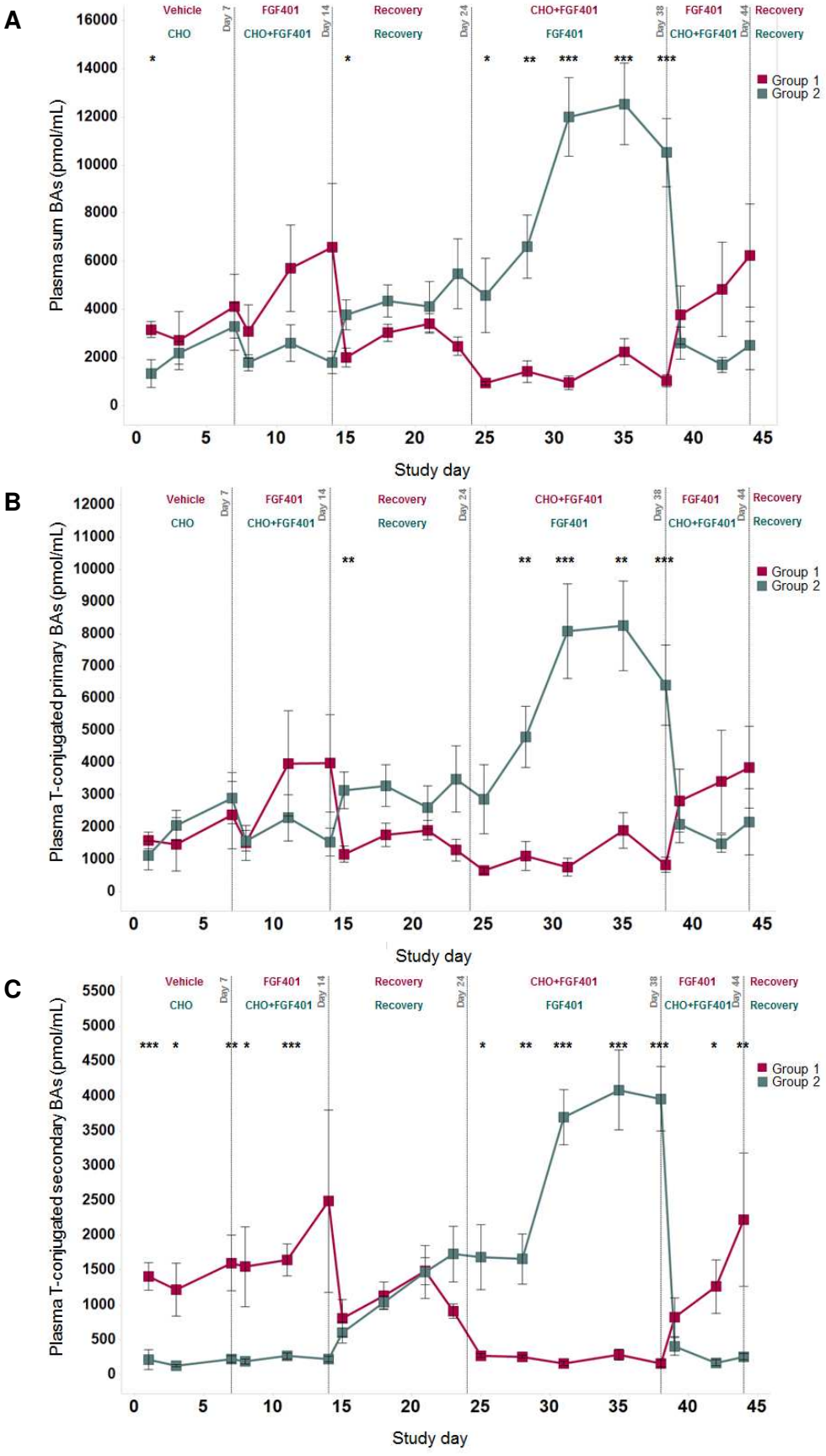


Figure 8: Changes in plasma BAs in investigative dog study with FGF401 and cholestyramine. (A) FGF401-mediated increases in plasma BAs at doses of 5 mg/kg/day could be mitigated and reversed by co-administration with the BA sequestrant CHO. (B) Primary T-conjugated (taurine) BAs in plasma (TCA, TCDCA). (C) Secondary T-conjugated BAs in plasma (TLCA, TDCA), for which CHO – mediated reductions appeared to be slightly more pronounced. N = 6 per group. Mean ± SEM are shown. *P < 0.05, ** < 0.01, and *** < 0.001, by students *t* test group 1 *versus* group 2.

Figure 9: Effect of FGF401 and cholestyramine on circulating cholesterol and C4. (A) Deceases in serum total cholesterol in dogs when treated with CHO or FGF401 alone, or under co-administration thereof. (B) Increases in plasma C4 in dogs when treated with CHO or FGF401 alone, indicating induction of hepatic Cyp7a1. Co-administration of CHO and FGF401 to dogs resulted in additional effects on plasma C4 elevation. N = 6 per group. Mean ± SEM are shown. *P < 0.05, ** < 0.01, and *** < 0.001, by students *t* test group 1 *versus* group 2.

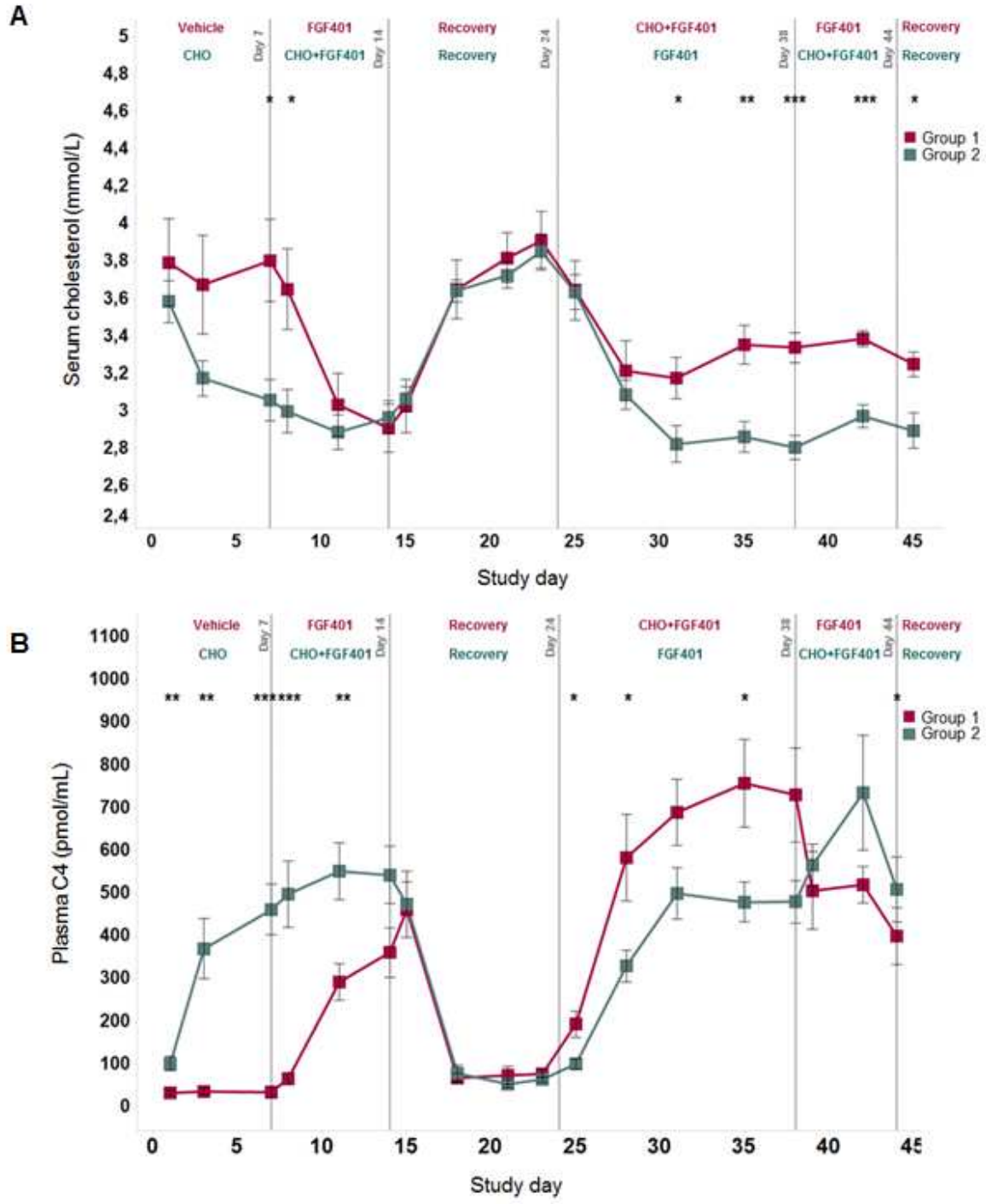
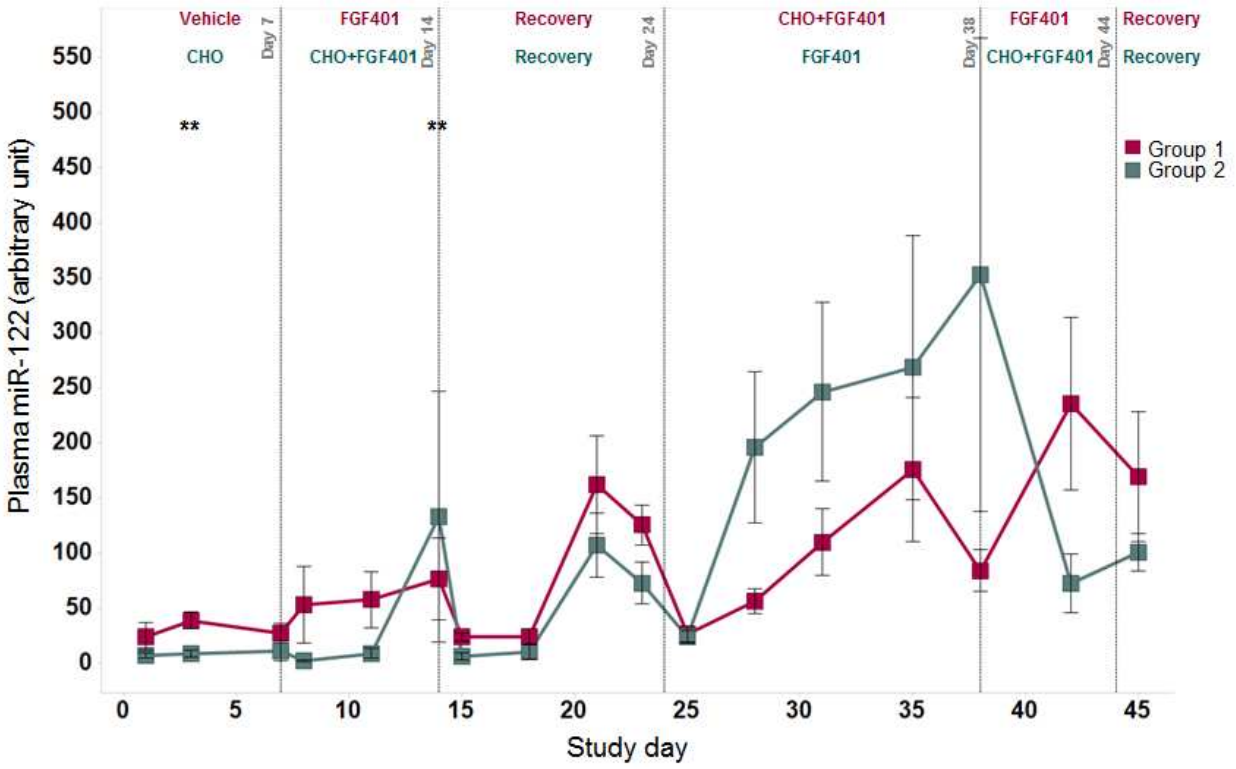


Figure 10: Circulating miR-122 as marker for hepatocellular injury. Plasma miR-122 in the investigative dog study with FGF401 and cholestyramine did not show consistent treatment-related changes, except for some minimal increases in individual dogs of Group 2 when treated with FGF401 alone (phase IV study days 25 to 38) which was reversed by co-treatment with CHO (phase V study days 39-44). N = 6 per group. Mean ± SEM are shown. ** P < 0.01 by students *t* test group 1 *versus* group 2



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